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Specific one-stage method for assay of enterokinase activity by release of radiolabelled activation peptides from α-*N*-[^{3}H]acetyl-trypsinogen and the effect of calcium ions on the enzyme activity

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We report a novel assay method for enterokinase capable of detecting approx. 1 fmol of enzyme. The method depends on quantification of the release of specifically radiolabelled activation peptides from bovine trypsinogen and is unaffected by trypsin inhibitors. The assay is applicable to biological fluids such as serum. The substrate was produced by selective ε-amidination of bovine trypsinogen followed by acetylation with [^{3}H]acetic anhydride and deprotection. The assay has been used to study the effects of pH, Ca^{2+}, ionic strength and glycodeoxycholate on enterokinase activity.

The conventional assay method for enterokinase (enteropeptidase, EC 3.4.21.9) is a two-stage procedure involving activation of trypsinogen followed by measurement of the trypsin activity formed with the use of low-molecular-weight synthetic substrates (Baratti *et al.*, 1973; Preiser *et al.*, 1975). The sensitivity of this assay method is increased by the amplification effect of a second catalytic event, but it has two principal disadvantages; it is unsatisfactory for the measurement of enterokinase activity in biological fluids containing trypsin inhibitors, and it has to be performed at pH<6 to prevent trypsinogen auto-activation, which interferes with detailed kinetic studies. More recently a one-stage assay method has been developed, with the use of Gly-[Asp]$_4$-Lys-2-naphthylamide (glycyl-tetra-L-aspartyl-L-lysine 2-naphthylamide), which overcomes the first of these constraints (Grant & Hermon-Taylor, 1979; Grant *et al.*, 1979). Although this synthetic substrate exploits the unusual tetra-aspartyl sequence common to mammalian trypsinogens, it cannot be used to determine the kinetic parameters of the interaction of enterokinase with its single natural substrate trypsinogen. Isotopic radiolabelling of the activation peptides of trypsinogen appeared to offer an alternative system. Sensitivity would be conferred by high specific radioactivity of the equimolar product, and independence of trypsin inhibitors would widen the scope of its practical application. The present paper reports the development of an alternative one-stage assay method for enterokinase with the use of trypsinogens as substrate, but based on detection of the release of specifically radiolabelled activation peptides rather than determination of the activity of the active trypsin formed.

Materials and methods

Materials

Bovine trypsinogen (2 × crystallized) and soya-bean trypsin inhibitor were from Sigma Chemical Co., Poole, Dorset, U.K. Methyl acetimidate was from Pierce and Warriner, Chester, Cheshire, U.K. CM-cellulose (CM-52) and 3MM chromatography paper were from Whatman Biochemicals, Maidstone, Kent, U.K. [^{3}H]Acetic anhydride (5.3 Ci/mmol) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were AnalaR to the purest grade available from BDH Chemicals, Poole, Dorset, U.K.

Human enterokinase was prepared by affinity chromatography, as previously described (Grant & Hermon-Taylor, 1976; Grant *et al.*, 1978). One unit of enzyme activity was defined as that amount of enzyme producing 1 nmol of active trypsin/min at 30°C. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Preparation of cationic bovine trypsinogen

A 500 mg portion of bovine trypsinogen was dissolved in 50 ml 10 mM-citric acid/NaOH buffer, pH 3.7, and applied to a CM-cellulose column (2.54 cm × 20 cm) pre-equilibrated in the same

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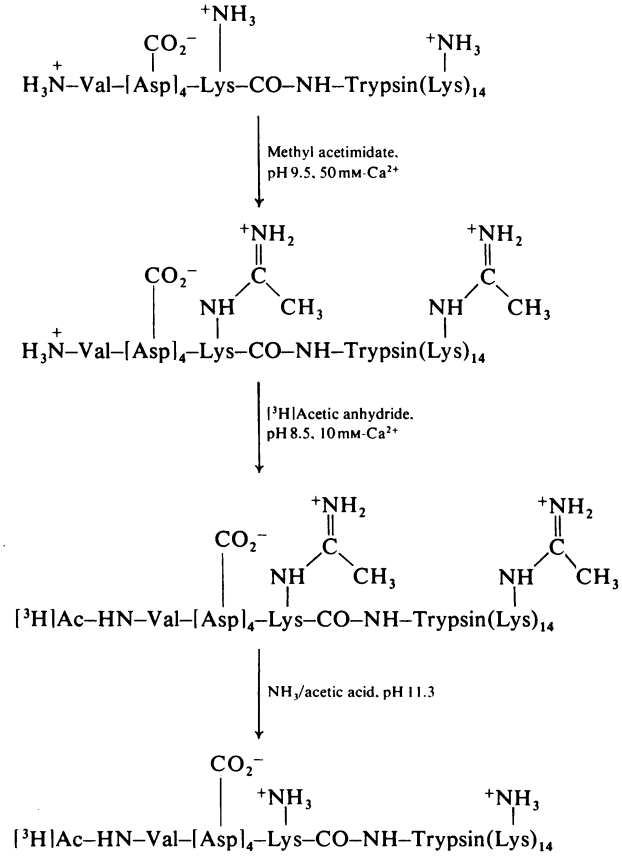
buffer. The column was washed and eluted with a linear gradient of 10–250mM-sodium citrate buffer, pH3.7, from two 200ml reservoirs. The eluted trypsinogen was dialysed against 1mM-HCl and freeze-dried. The pure zymogen gave one major band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and an amino acid composition in agreement with that previously described (Walsh & Neurath, 1964).

Amidination and α-N-[^{3}H]acetylation of trypsinogen

Preliminary studies showed that specific *N*-terminal tritiation to produce a product that could be hydrolysed by enterokinase could only be achieved by selective protection of the lysine ε-amino groups, followed by specific acetylation of the *N*-terminal α-amino group and subsequent deprotection. Methyl acetimidate (Hunter & Ludwig, 1962) has been shown to react reasonably selectively with ε-amino groups at pH9.5 and above, and is removable by treatment with aq. NH_3 (sp.gr.0.880)/acetic acid (15:1, v/v), pH11.3 (Ludwig & Byrne, 1962). To determine whether trypsinogen would withstand these conditions, the zymogen was first incubated in the deprotection reagent for 6h, followed by dilution and freeze-drying. The product was found to retain >80% of its expected tryptic activity after activation by enterokinase.

The procedure for acetylation of the α-amino group of trypsinogen with [^{3}H]acetic anhydride is summarized in Scheme 1. Selective blocking of ε-amino groups was performed essentially by the method of Nureddin & Inagami (1969). A 100mg portion of purified trypsinogen (1mg/ml in 50mM-$CaCl_2$) was adjusted to pH9.5 with 2M-NaOH, and 220mg of solid methyl acetimidate was added at room temperature, the pH being maintained at 9.5 by titration with 2M-NaOH. Five more 220mg portions of methyl acetimidate were added at 20min intervals. The reaction was terminated after 3h by titration to pH3.0 with 1M-HCl and the product was dialysed against 1mM-HCl and freeze-dried. Amino



Scheme 1. *Semi-synthetic route to α-N-[^{3}H]acetyl-trypsinogen*
For full experimental details see the text.

acid analysis after 24h, 48h and 72h hydrolyses and extrapolation of the free lysine content to $t = 0$ showed that 16.03 mol of lysine residues had been amidinated per mol of trypsinogen.

The protected trypsinogen (92mg) was dissolved in 10ml of 0.1M-boric acid/NaOH buffer, pH8.5. To this was added 25mCi of [^{3}H]acetic anhydride dissolved in 2ml of acetonitrile. The mixture was stirred at room temperature for 1h, dialysed against several 1-litre changes of 1mM-HCl and freeze-dried.

The protecting groups were removed by dissolving the labelled protein in 5ml of aq. NH_3 (sp.gr.0.880)/acetic acid (15:1, v/v), pH11.3. After 6h at room temperature the mixture was diluted with 50ml of 20mM-$CaCl_2$ and freeze-dried. The residue was redissolved in 50ml of water and again freeze-dried. The product was taken up in 20ml of 10mM-sodium citrate buffer, pH3.7, and chromatographed on CM-cellulose as described above. The column was washed with 1.5 column volumes of buffer; tritiated trypsinogen was then recovered as a single symmetrical peak by gradient elution as described above. The specific radioactivity of the α-*N*-[^{3}H]acetylated trypsinogen was 0.58Ci/mmol (24.2μCi/mg; 2.15×10^7c.p.m./mg), which corresponds to an average substitution of 0.23mol of [^{3}H]acetyl group/mol of zymogen.

A 50μl portion of the tritiated trypsinogen solution (approx. 10^5c.p.m.) was incubated with 200μg of soya-bean trypsin inhibitor with or without 85μunits of purified human enterokinase (Grant & Hermon-Taylor, 1976) in a final volume of 0.4ml of 20mM-Tris/20mM-sodium maleate buffer, pH8.0, containing 10mM-$CaCl_2$. In a separate experiment 50μl of the tritiated zymogen solution was incubated with 5μg of bovine trypsin in the same buffer but without soya-bean trypsin inhibitor. After 16h at room temperature, 0.2ml of 25% (w/v) trichloroacetic acid was added and the sample was left overnight. The pellet was spun down and a 50μl sample of the supernatant was analysed by high-voltage paper electrophoresis on Whatman 3MM paper at pH6.5 in pyridine/acetic acid/water (25:1:225, by vol.). After 90min at 3000V the electrophoretograms were stained with ninhydrin (Dreyer & Bynum, 1967). The relative mobilities of ninhydrin-positive spots were expressed relative to the migration of aspartic acid. Displacement of the origin due to electroendosmosis was determined by using *N*-2,4-dinitrophenylethanolamine. The electrophoretogram was cut into 2cm × 1cm pieces, the radioactivity of each of which was counted in 5ml of scintillant stock solution [made up from 1 litre of toluene, 0.5g of 2,5-diphenyloxazole and 0.05g of 1,4-bis-(5-phenyloxazol-2-yl)benzene] in an SL-30 Intertechnique liquid-scintillation counter.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in 11.5% gels as described by Neville (1971). Isoelectric focusing was performed as described by the manufacturer on pre-cast polyacrylamide gels (LKB Instruments, Croydon, Surrey, U.K.) in a pH3–10 Ampholine gradient. Radioactive bands on gels were detected by fluorography (Laskey & Mills, 1975).

Optimum pH and Ca^{2+} concentration for hydrolysis of α-N-[^{3}H]acetyl-trypsinogen by enterokinase: effects of glycodeoxycholate and ionic strength

Buffers in the range pH5–10 were made up from 20mM-Tris/20mM-maleic acid/20mM-glycine by adjusting the pH with NaOH or HCl. Approx. 0.05μCi of α-*N*-[^{3}H]acetyl-trypsinogen was incubated for 90min at 30°C in 0.2ml of buffer with 40μg of soya-bean trypsin inhibitor and 12μunits of human enterokinase (Grant *et al.*, 1978). Then 100μl of 25% (w/v) trichloroacetic acid was added and each sample was left at 4°C overnight. After removal of the precipitate by centrifugation, the radioactivity of a 100μl sample of the supernatant was counted in 5ml of stock scintillant solution [made up from 1 litre of toluene, 500ml of Triton X-100, 0.5g of 2,5-diphenyloxazole and 0.05g of 1,4-bis-(5-phenyloxazol-2-yl)benzene]. The effect of inclusion of Ca^{2+} (0–15mM) in the buffer was also studied. The effect of glycodeoxycholic acid (0–10mM) on the hydrolysis of the zymogen by enterokinase was tested in a further series of experiments. Ionic strength was varied by making the incubation mixture 0–250mM with respect to NaCl.

Kinetic study of the enzymic reaction

The specific radioactivity of α-*N*-[^{3}H]acetyl-trypsinogen was adjusted to 0.025μCi/mg by the addition of unlabelled bovine trypsinogen. Various amounts of radiolabelled substrate were added to 0.2ml of 20mM-Tris/20mM-sodium maleate buffer, pH8.0, containing 7.5mM-$CaCl_2$ and 200μg of soya-bean trypsin inhibitor/ml, so that the final concentration of zymogen was in the range 7.5–225μM. After incubation at 30°C for 90min, the hydrolysis was stopped by the addition of 100μl of 25% (w/v) trichloroacetic acid, and 100μl of acid-soluble material was separated and its radioactivity counted as described above. The data were plotted in the form $[S]/v$ against $[S]$, where v represents the initial velocity (Hanes, 1932).

Assay of enterokinase in serum

A 10μl portion of human serum to which had been added 1–7 units of human enterokinase was mixed with 0.15ml of 20mM-Tris/20mM-sodium maleate buffer, pH8.0, containing 10mM-$CaCl_2$. Then 40μg of soya-bean trypsin inhibitor in 20μl of water was added, followed by 0.05μCi of α-*N*-[^{3}H]acetyl-trypsinogen (specific radioactivity

24 μCi/mg) in 20 μl of 1 mM-HCl. After incubation at 30°C for 90 min the reaction was stopped by the addition of 0.1 ml 25% (w/v) trichloroacetic acid and the mixture was left overnight at 4°C. After centrifugation, the radioactivity of the supernatant was counted as described above.

Results

The α-*N*-[^{3}H]acetyl-trypsinogen co-electrophoresed with native bovine trypsinogen in sodium dodecyl sulphate/11.5% polyacrylamide gels. Subsequent fluorography showed a single band that was coincident with a Coomassie Blue-stained band corresponding to unlabelled trypsinogen. Isoelectric focusing in the pH range 3–10 also showed co-focusing between the native and the modified zymogen.

The electrophoretogram of trichloroacetic acid-soluble α-*N*-[^{3}H]acetyl activation peptides is shown in Fig. 1. Hydrolysis of the labelled zymogen by enterokinase released over 75% of the total radioactivity in 16 h and showed a major peak of radioactivity with a relative mobility of −1.02 and a second minor peak at −0.81. No other peaks of radioactivity with anodal or cathodal mobilities were seen. Less than 20% of the acid-soluble radioactivity remained at the origin.

In the absence of Ca^{2+}, release of trichloroacetic acid-soluble radioactivity from α-*N*-[^{3}H]acetyl-trypsinogen by enterokinase was greatest at pH 6. The pH optimum was, however, dependent on the Ca^{2+} concentration (Fig. 2). Maximum activity was seen in 20 mM-Tris/20 mM-sodium maleate buffer, pH 8.0, containing 7.5 mM-$CaCl_2$ final concentration. At this pH the presence of 7.5 mM-Ca^{2+} increased hydrolysis of the unlabelled zymogen by enterokinase approximately 3-fold over that seen in the absence of the cation, and subsequent assays were performed in this buffer. The addition of 50 mM-NaCl decreased enterokinase activity by approx. 80%, and activity was virtually abolished at concentrations of 100 mM-NaCl and above. An apparent stimulation of

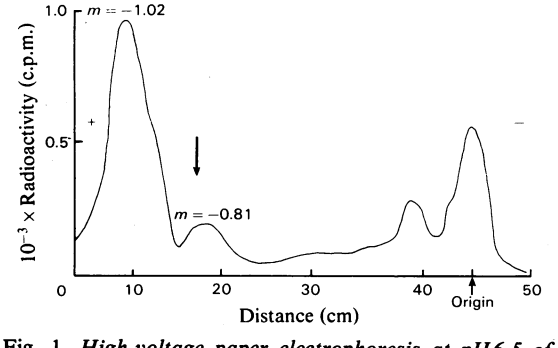


Fig. 1. *High-voltage paper electrophoresis at pH 6.5 of released α-N-[^{3}H]acetyl activation peptides*
For full experimental details see the text. Mobilities are given relative to aspartic acid ($m = -1.0$) and *N*-2,4-dinitrophenylethanolamine ($m = 0$). The arrow shows the migration position of unacetylated Val-[Asp]$_4$-Lys.

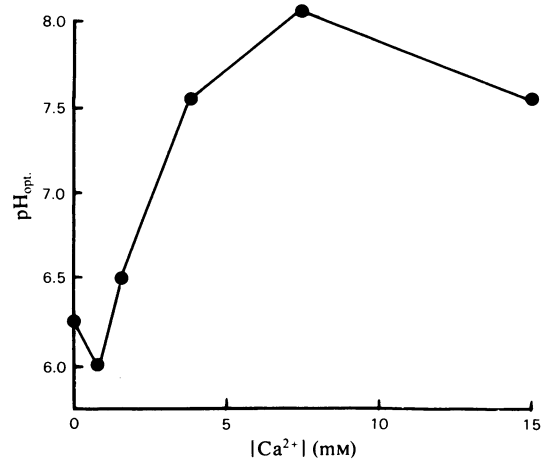


Fig. 2. *Relationship between pH optimum and Ca^{2+} concentration in the hydrolysis of α-N-[^{3}H]acetyl-trypsinogen by enterokinase*
For full experimental details see the text.

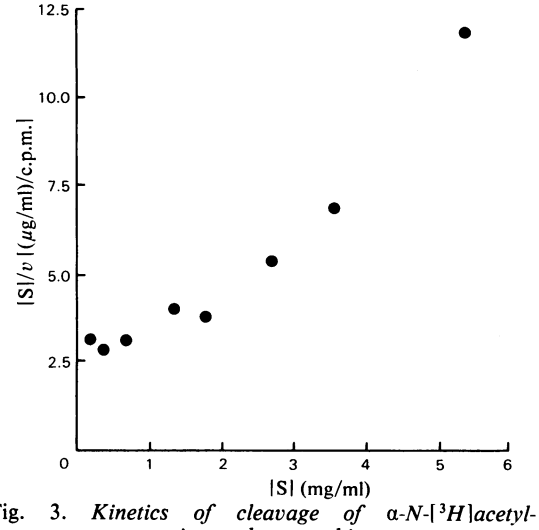


Fig. 3. *Kinetics of cleavage of α-N-[^{3}H]acetyl-trypsinogen by enterokinase*
Hanes plot of the dependence of initial rate of cleavage (v = c.p.m. released/90 min per 100 μl of supernatant) on substrate concentration ([S] = trypsinogen concentration in mg of protein/ml).

enterokinase activity in the presence of 2.5mM-glycodeoxycholate was found to be an artifact due to the fact that detergent flocculation that followed the addition of trichloroacetic acid entrapped labelled substrate in the supernatant.

The Hanes plot ([S]/v against [S]) is shown in Fig. 3. Values for K_m and $V_{max.}$ could not be estimated, because of its non-linearity. The amount of α-*N*-[^{3}H]acetyl-trypsinogen hydrolysed was directly proportional to the amount of exogenous enterokinase mixed with serum; the limit of detection was about 0.1ng of enzyme protein (5–10ng/ml) of serum), equivalent to approx. 1fmol of enterokinase.

Discussion

We have devised a sensitive and specific assay method for enterokinase based on the release of radiolabelled activation peptides from bovine trypsinogen. This assay method has the advantages over previous two-stage assays that it can be performed in the presence of trypsin inhibitors, e.g. in serum, and at the optimum pH and Ca^{2+} concentration for enterokinase activity.

Specific *N*-terminal acetylation with [^{3}H]acetic anhydride by attempting to exploit the differences in pK between the α-amino group and the ε-amino groups of the 15 constituent lysine residues of trypsinogen was unsuccessful, since there was little release of α-*N*-[^{3}H]acetyl activation peptide after incubation with enterokinase. It was clear that selective protection of ε-amino groups was necessary. Anderson *et al.* (1977) reported that guanidinated bovine trypsinogen was activated by bovine enterokinase at about 30% of the rate of the native zymogen, despite guanidination of the lysine residue on the carbonyl side of the susceptible lysyl-isoleucyl bond. Preliminary experiments (A. I. Magee, D. A. W. Grant & J. Hermon-Taylor, unpublished work) showed that activation of guanidinated bovine trypsinogen by human enterokinase was diminished to less than 1% of the normal rate, and it was apparent that protection of ε-amino groups had to be reversible. Citraconylation, reversible at pH3, was not selective for the ε-amino groups of bovine trypsinogen; sufficient selectivity was achieved by using methyl acetimidate at pH9.5, leaving α-amino groups free for subsequent [^{3}H]acetylation. The ability of bovine trypsinogen to resist the harsh conditions of deprotection may reflect stabilization of the molecule by its five disulphide linkages and its capacity to survive the environment of the duodenal lumen.

Webster & Offord (1977) demonstrated the release of two activation peptides from bovine cationic trypsin; Val-[Asp]$_4$-Lys (valyl-tetra-aspartyl-lysine) was the major peptide and Phe-Pro-Val-[Asp]$_4$-Lys (phenylalanyl-prolyl-valyl-tetra-aspartyl-lysine) the minor form. These findings are supported by the present study; the observed electrophoretic mobilities were in agreement with those that would be predicted (Offord, 1966) on the basis of acetylation of the α-amino group with an increase in net negative charge.

The concentration of Ca^{2+} required for maximum enterokinase activity depends on the pH. We found that in the presence of an excess of trypsin inhibitor, but in the absence of Ca^{2+}, the pH optimum for the activity of human enterokinase (pH6) is in agreement with the findings obtained by Rinderknecht *et al.* (1974) with the unlabelled substrate. The pH optimum of 8 in 7.5mM-Ca^{2+} corresponds to that found with the artificial substrate Gly-[Asp]$_4$-Lys-2-naphthylamide (Grant & Hermon-Taylor, 1979). In the assay system reported in the present paper enterokinase is approximately twice as active at pH6 than at pH8 in the absence of Ca^{2+}, but only about 60% as active in the presence of 7.5mM-Ca^{2+}. The relative independence of enterokinase on [Ca^{2+}] at pH6 would favour enzyme activity in the duodenal lumen; the requirement for Ca^{2+} at higher pH would impede the inappropriate activation of trypsinogen if enterokinase gained access to the pancreatic ducts, where the pH is more than 8 and the Ca^{2+} concentration less than 2mM. Although an increase in enterokinase activity by glycodeoxycholate has been suggested by studies with isolated enterocyte microvilli (Hadorn *et al.*, 1974), no comparable stimulation was found for the free enzyme against trypsinogen in the one-stage assay procedure.

The presence of an excess of soya-bean trypsin inhibitor in the incubation mixture enabled the kinetics of enterokinase cleavage of trypsinogen to be studied at high substrate concentration. At these concentrations (above 2–3mg/ml) a phenomenon resembling substrate inhibition occurred. Such inhibition could be due to non-productive binding of the substrate to enterokinase, as observed by Rinderknecht & Friedman (1978).

The labelled zymogen has provided a satisfactory alternative to the artificial substrate Gly-[Asp]$_4$-Lys-2-naphthylamide (Grant & Hermon-Taylor, 1979) in the assay of enterokinase in biological fluids, with a detection limit less than 0.5ng of enterokinase protein. There is, in contrast with the Gly-[Asp]$_4$-Lys-β-naphthylamidase assay method, no interference by arylamidases, and specificity can be confirmed by using anti-enterokinase antibody, which inhibits trypsinogen activation but not the hydrolysis of the low-molecular-weight artificial substrate (Grant & Hermon-Taylor, 1979). The labelled zymogen has been used to detect both human and pig enterokinases in hepatic bile after intravenous administration in rats (Grant *et al.*, 1980). Since the interference from bacterial amino-

acylases (Chibata *et al.*, 1972) and deacetylases may occur, the specific release of labelled activation peptides should be checked by high-voltage paper electrophoresis when the assay method is first applied to a system in which the samples are potentially contaminated with these enzymes.

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References

Anderson, L. E., Walsh, K. A. & Neurath, H. (1977) *Biochemistry* **16**, 3354–3360

Baratti, J., Maroux, S. & Louvard, D. (1973) *Biochim. Biophys. Acta* **321**, 632–638

Chibata, I., Tosa, T., Sato, T., Mori, T. & Matsuo, Y. (1972) *Proc. Meet. Int. Ferment. Soc. 4th* 383–389

Dreyer, W. J. & Bynum, E. (1967) *Methods Enzymol.* **11**, 32–39

Grant, D. A. W. & Hermon-Taylor, J. (1976) *Biochem. J.* **155**, 243–254

Grant, D. A. W. & Hermon-Taylor, J. (1979) *Biochim. Biophys. Acta* **567**, 207–215

Grant, D. A. W., Magee, A. I. & Hermon-Taylor, J. (1978) *Eur. J. Biochem.* **88**, 183–189

Grant, D. A. W., Magee, A. I., Meeks, D., Regan, C., Bainbridge, D. R. & Hermon-Taylor, J. (1979) *Biochem. J.* **184**, 619–626

Grant, D. A. W., Jones, P. A., Magee, A. I. & Hermon-Taylor, J. (1980) *Biochem. Soc. Trans.* **8**, 55

Hadorn, B., Hess, J., Troesch, V., Verhaage, W., Gotze, H. & Bender, S. W. (1974) *Gastroenterology* **66**, 548–555

Hanes, C. S. (1932) *Biochem. J.* **26**, 1406–1421

Hunter, M. J. & Ludwig, M. L. (1962) *J. Am. Chem. Soc.* **84**, 3491–3504

Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Ludwig, M. L. & Byrne, R. (1962) *J. Am. Chem. Soc.* **84**, 4160–4162

Neville, D. M., Jr. (1971) *J. Biol. Chem.* **246**, 6328–6334

Nureddin, A. & Inagami, T. (1969) *Biochem. Biophys. Res. Commun.* **36**, 999–1005

Offord, R. E. (1966) *Nature (London)* **211**, 591–593

Preiser, H., Schmitz, J., Maestracci, D. & Crane, R. K. (1975) *Clin. Chim. Acta* **59**, 169–175

Rinderknecht, H. & Friedman, R. M. (1978) *Biochim. Biophys. Acta* **525**, 200–208

Rinderknecht, H., Engeling, E. R., Bunnell, M. J. & Geokas, M. C. (1974) *Clin. Chim. Acta* **54**, 145–160

Walsh, K. & Neurath, H. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 884–889

Webster, D. & Offord, R. E. (1977) *Biochem. J.* **167**, 285–288